स्तन कैंसर के लिए पूर्वानुमान कारक चिह्नक

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स्तन कैंसर रोगियों में पुनरावृत्ति, मेटास्टेसिस और चिकित्सा अनुक्रिया के लिए रोगसूचक और पूर्वानुमानित चिह्नक के रूप में थायोरेडॉक्सिन रिडक्टेस 1

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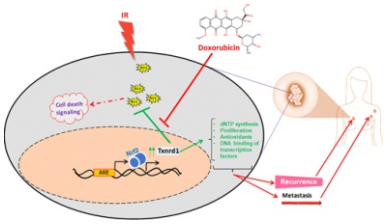

Txnrd1 अति-अभिव्यक्ति स्तन कैंसर में पुनरावृत्ति, मेटास्टेसिस और चिकित्सा प्रतिक्रिया से जुड़ी हुई है।

सारांश

थायोरडॉक्सिन रिडक्टेस 1 (Txnrd1) को स्तन कैंसर रोगियों के एक उपसमूह में रोगनिदान संबंधी महत्व के लिए जाना जाता है। Txnrd1 को कैंसर की प्रगति और मेटास्टेसिस में कई कोशिकीय एवं शारीरिक प्रक्रियाओं को विनियमित करने में एक महत्वपूर्ण भूमिका निभाने के लिए जाना जाता है, हालाँकि, इसका नैदानिक महत्व काफी हद तक पहचाना नहीं गया है। यहाँ, Txnrd1 की रोगनिदान संबंधी और भविष्य निर्धारण की भूमिका का आकलन करने के लिए 43 स्वतंत्र समूहों से 13322 स्तन कैंसर रोगियों का पूर्वव्यापी व्यापक मेटा-विश्लेषण किया गया। यह पाया गया कि Txnrd1 का ट्यूमर ग्रेड और आकार के साथ सकारात्मक संबंध है। इसके अलावा, नकारात्मक-रिसेप्टर और सकारात्मक-HER2 हार्मोन ट्यूमर में Txnrd1 जीन अधिक अनुकूल होती है। उच्च Txnrd1 अनुकूलन वाले रोगी लघु रोग-वैशिष्ट्य और समग्र उत्तरजीविता के लिए महत्वपूर्ण जोखिम प्रदर्शित करते हैं। जबकि, Txnrd1 का ट्यूमर पुनरावृत्ति और मेटास्टेसिस के साथ सकारात्मक सहसंबंध है, तथापि इसकी पुनरावृत्ति और मेटास्टेसिस के समय के साथ नकारात्मक संबंध है। Txnrd1 उच्च रोगियों में Txnrd1 निम्न समूह की तुलना में 2.5 वर्ष पहले पुनरावृत्ति और 1.3 वर्ष पहले मेटास्टेसिस होता है। दिलचस्प बात यह है कि उच्च Txnrd1 जीन अभिव्यक्ति वाले रोगी नियोएडजुवेंट कीमोथेरेपी के लिए एक पैथोलॉजिकल पूर्ण अनुक्रिया (pCR) प्रदर्शित करते हैं, लेकिन रेडियोथेरेपी के बाद, वे जल्दी पुनरावृत्ति का अनुभव करते हैं। Txnrd1 उच्च MDA-MB-231 कोशिकाएं Txnrd1 निम्न MCF7 कोशिकाओं की तुलना में डॉक्सोरूबिसिन उपचार के बाद महत्वपूर्ण ROS उत्पादन और कम व्यवहार्यता प्रदर्शित करती हैं। मेटा-विश्लेषण से प्राप्त निष्कर्षों की पुष्टि करते हुए, Txnrd१ की कमी से जीवित रहने की दर में कमी, विकिरण प्रेरित हत्या के प्रति संवेदनशीलता में वृद्धि, खरोंच-घाव का खराब उपचार और MDA-MB-231 कोशिकाओं में आक्रमण की संभावना कम हो जाती है। इस प्रकार, Txnrd1 स्तन कैंसर रोगियों में पुनरावृत्ति, मेटास्टेसिस और चिकित्सा अनुक्रिया से संबंधित भविष्य की संभावित जानकारी प्रदान करता प्रतीत होता है।

Predictive markers for Breast Cancer

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Thioredoxin Reductase 1 as a Prognostic & Predictive Marker for Recurrence, Metastasis & Therapy Response in Breast Cancer Patients

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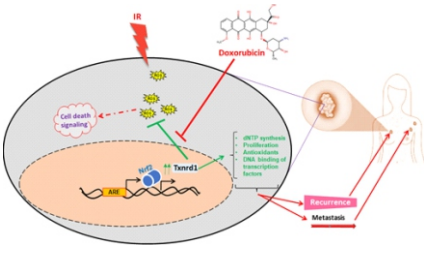

Txnrd1 over-expression is associated with recurrence, metastasis and therapy response in breast cancer

ABSTRACT

Thioredoxin reductase 1 (Txnrd1) is known to have prognostic significance in a subset of breast cancer patients. Txnrd1 is known to play a pivotal role in regulating several cellular and physiological processes in cancer progression and metastasis, however, its clinical significance is largely unrecognized. Here, a retrospective comprehensive meta-analysis of 13322 breast cancer patients from 43 independent cohorts to assess prognostic and predictive roleof Txnrd1 was undertaken. It was observed that Txnrd1 has a positive correlation with tumor grade and size. Further, hormone receptor-negative and HER2-positive tumors exhibit elevated Txnrd1 gene expression. Patients with elevated Txnrd1 expression exhibit significant hazards for shorter disease-specific and overall survival. While Txnrd1 has a positive correlation with tumor recurrence and metastasis, it has a negative correlation with time for recurrence and metastasis. $Txnrd1^{High}$ patients exhibit 2.5 years early recurrence and 1.3 years early metastasis as compared to $Txnrd1^{Low}$ cohort. Interestingly, patients with high Txnrd1 gene expression exhibit a pathologic complete response (pCR) to neoadjuvant chemotherapy, but they experience early recurrence after radiotherapy. $Txnrd1^{High}$ MDA-MB-231 cells exhibit significant ROS generation and reduced viability after doxorubicin treatment compared to $Txnrd1^{Low}$ MCF7 cells. Corroborating with the findings from meta-analysis, Txnrd1 depletion leads to decreased survival, enhanced sensitivity to radiation induced killing, poor scratch-wound healing, and reduced invasion potential in MDA-MB-231 cells. Thus, Txnrd1 appears to be a potential predictor of recurrence, metastasis and therapy response in breast cancer patients.

KEYWORDS: *Thioredoxin reductase, Nrf2, ROS, Ionizing radiation, Therapy response*

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Introduction

Breast cancer is a complex disease characterized by four major molecular subtypes and 21 distinct histological subtypes, with approximately 81% being invasive and 83% hormone receptor-positive, while around 15% are HER2-positive [1]. The age-standardized incidence and mortality rate for breast cancer are 47.8 and 13.6 per 100,000 cases, respectively [2]. Despite advancements in treatment modalities and early detection, there is an increase in the incidence of local-stage disease, though mortality rates have declined significantly [3]. Treatment modalities like chemo, hormonal, or targeted molecular therapy have shown promising results in locally advanced breast cancers (LABC), triple-negative breast cancer (TNBC), and metastatic disease when combined with surgery [4]. However, there is still a risk of recurrence, reduced disease-free survival (DFS) and poor quality of life (QoL) among survivors [5]. Different factorcontribute to breast cancer recurrence include tumor size, lymph node involvement, close tumor margins, age, lack of hormone and radiotherapy, and inflammatory breast cancer [6]. In addition to traditional prognostic factors, gene expression profiling plays a crucial role in prognosis and treatment planning. Radiotherapy post-breast-conserving surgery aims to eliminate remnant cancer cells [7], yet intrinsic radio-resistance and the acquisition of a resistant phenotype limit its efficacy, necessitating predictive tools for treatment decisions.

Studies from our laboratory have highlighted the role of Nrf2 and oxidative stress response pathways in regulating radio-resistance in cancer cells [8]. Thioredoxin reductase (TrxR), controlled by Nrf2, is a key redox-regulatory protein involved in various cellular processes. Overexpression of Txnrd1, a subtype of TrxR, has been associated with several cancers, including breast cancer, impacting invasion, metastasis, and prognosis [9, 10]. Given its significance, a detailed investigation across global datasets is warranted to understand its predictive value for recurrence, metastasis, and treatment response. A retrospective analysis was conducted, encompassing RNAseq and microarray data from 13,322 breast cancer patients across 43 independent gene expression datasets. The study examined the association of Txnrd1 expression with various clinic-pathological parameters, including tumor grade, size, stage, histology, menopause status, molecular subtypes, overall survival (OS), disease-specific survival (DSS), recurrence, metastasis, and response to chemotherapy and radiotherapy. Experimental validation in breast cancer cell lines further supported Txnrd1 as a valuable predictor for disease recurrence and therapy response in breast cancer patients.

Materials and Methods

Reagents

The human breast cancer cell lines MCF-7, MDA-MB468, and MDA-MB-231 were obtained from the National Centre for Cell Science (NCCS, Pune, India) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. Reagents used in the study, including the thioredoxin reductase assay kit, RNA isolation kit, cDNA synthesis, RT-PCR kit, doxorubicin, auranofin, formaldehyde, crystal violet, H_2DCF-DA, TrxR1 CRISPR plasmids, and Lipofectamine 3000 transfection reagent, were procured from various suppliers.

Study design and gene expression data

A retrospective meta-analysis was conducted using gene expression data from 43 public datasets, comprising microarray/RNAseq analysis of tumor specimens from 13,322 breast cancer patients. Data sources included the NCBI Gene Expression Omnibus (GEO), Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database, The Cancer Genome Atlas (TCGA), and other sources. Optimal cut-off points for Txnrd1 gene expression were determined using various methods, and patients were categorized into Txnrd1^{High} and Txnrd1^{Low} subgroups accordingly.

Study endpoints

Study endpoints were divided into three categories: cross-study comparison, survival analysis, and meta-analysis. Cross-study comparison involved analyzing Txnrd1 gene expression across various clinic-pathological parameters. Survival analysis was conducted to determine cumulative mean time to outcome, while meta-analysis combined effect sizes across datasets using multivariate Cox-regression analysis.

In vitro assays

RNA isolation, cDNA synthesis, and qRT-PCR were conducted to analyze gene expression. Transfection of TrxR1 CRISPR/Cas9 KO Plasmid was performed using Lipofectamine 3000 reagent, and clonogenic, wound healing, and invasion assays were conducted to assess cellular functions.

Statistical analysis

Statistical analysis included estimation of differences in gene expression, correlation coefficient calculation, survival curve comparison, hazard ratio and relative risk estimation, and meta-analysis. Statistical significance was determined using t-tests, one-way ANOVA, log-rank tests, and other appropriate methods. Data were presented as mean ± S.E.M.

Results and Discussion

Txnrd1 expression exhibits positive correlation with tumor grade and size

The selection criteria for datasets and the corresponding cohort sizes are outlined in Fig.1(a), providing a visual representation of the dataset selection process. Txnrd1 mRNA levels are altered in ~14% breast cancer patients (Fig.1(b)). In 21 datasets analyzed, a significant variation in Txnrd1 gene expression according to tumor grade was observed, with higher expression correlating with more aggressive tumors (Fig.1(c)). Additionally, tumors with high Txnrd1 expression are larger by 2.59±0.8 mm (95% CI, 0.849-4.331; at $p < 0.007$) compared to those with low expression (Fig.1(d)). Hormone-receptor negative and HER2 positive tumors harbor elevated Txnrd1 expression (Fig.1(e)). Depletion of Txnrd1 in aggressive breast cancer cells reduced clonogenic potential, supporting its role in promoting cell survival and proliferation (Fig.1(f)). This association is particularly relevant for locally advanced breast cancers, where aggressive tumors exhibit elevated Txnrd1 expression, potentially influencing their prognosis. Further, mRNA expression corroborated well with respective breast cancer cell lines (Fig.1(g)).

Txnrd1 over-expressing breast cancer patients exhibit shorter disease-specific and overall survival:

***Abbreviations**: Auranofin, AF; DFS, disease-free survival; DSS, disease-specific survival; DRFS, distant recurrence-free survival; DMFS, distant metastasis-free survival; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; LABC, locally advanced breast cancer; MFI, metastasis-free interval; OS, overall survival; pCR, pathologic complete response; PFS, progression-free survival; PR, progesterone receptor; RD, residual disease; RCB, residual cancer burden; RFS, recurrence-free survival; ROS, reactive oxygen species; RR, relative risk; Trx, thioredoxin; TrxR, thioredoxin reductase; Txnrd1, thioredoxin reductase 1; TNBC, triple-negative breast cancer; QoL: quality of life;*

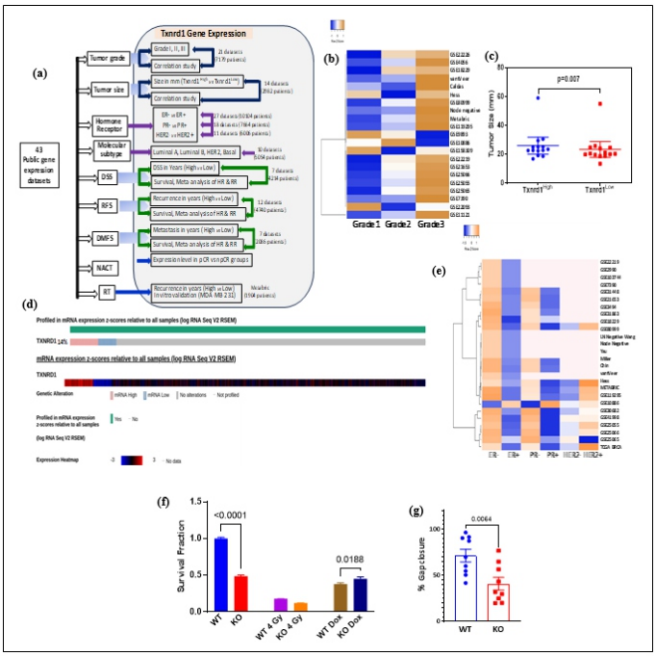


Fig.1: (a) Workflow outlining the strategy employed for analysis of public gene expression data with respect to Txnrd1 gene expression, (b) mRNA expression z-scores relative to all samples from TCGA-Firehose Legacy database with expression heatmap obtained from cBioportal, (c) The expression heatmap of Txnrd1 across tumor grades from different datasets with row z-scores and clustering, (d) The mean tumor size in Txnrd1 High and Low expression subgroups, (e) Expression heatmap of Txnrd1 across ER, PR & HER2 expression status along with row z-scores and clustering. Adapted from Patwardhan et al., Heliyon, 2024. 10(6):e27011

Txnrd1 over-expression is associated with poorer breast cancer-specific survival, as demonstrated in a combined analysis of seven datasets, where Txnrd1^{High} patients exhibited disease-related mortality ~2.03 years earlier than Txnrd1^{Low} patients (Fig.2(a)). Additionally, overall survival analysis across ten datasets revealed significantly shorter (~2 years) survival for patients with Txnrd1 over-expression (Fig.2(b)). Despite some variability across datasets, the overall findings underscore the adverse prognostic impact of Txnrd1 over-expression on breast cancer survival.

Breast cancer patients with over-expression of Txnrd1 are at elevated risk of early recurrence and metastasis

Txnrd1^{High} patients experienced ~2.5 years earlier disease recurrence than Txnrd1^{Low} group (Fig.2(c)). Interestingly, findings from meta-analysis coincided with combined survival analysis illustrating a statistically significant hazard (pooled hazard ratio1.47, p<0.001) of early recurrence in Txnrd1 over-expressing breast cancer patients (Fig.2(d)). The robustness of these findings is supported by very low associated heterogeneity and between-study variance. Moreover, meta-analysis of log-transformed relative risks identified a statistically significant positive risk of early recurrence in Txnrd1^{High} breast cancer patients. Txnrd1^{High} breast cancer patients showed statistically significant hazard for the shorter metastasis-free period and an elevated risk for early metastasis events (Fig.2(e)). Validation experiments revealed that Txnrd1 depletion in Txnrd1^{High} MDA-MB-231 cells abrogated the motility compared to wild type cells (Fig.3(e)). Concomitantly, pharmacologic inhibition of Txnrd1 hampered the invasiveness of MDA-MB-231 cells (Fig. 3(f)), supporting

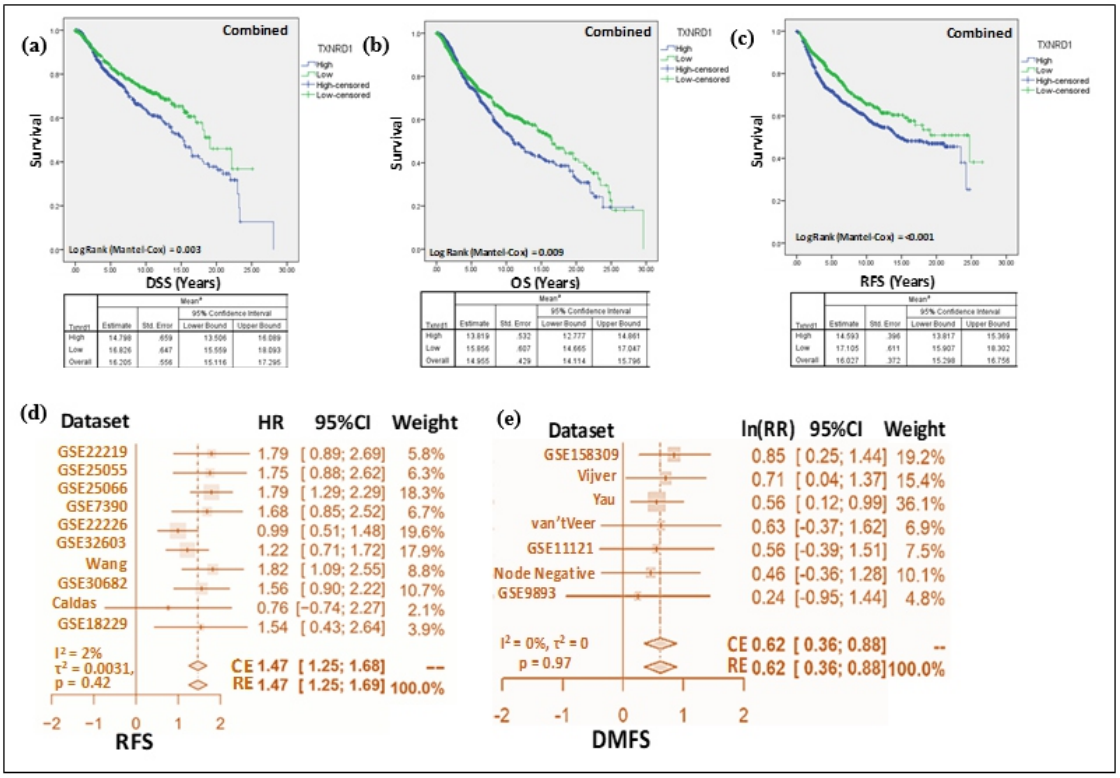


Fig.2: (a) Kaplan-Meier survival curve for disease-specific survival generated by pooling the quantile normalized data from all the datasets along with the Log-Rank (Mantel-Cox) test p-value. The cumulative mean survival time between high (n=722) and low (n=714) expression subgroups is shown below, (b) Kaplan-Meier survival curve for overall survival generated by pooling the quantile normalized data from all the datasets along with Log-Rank (Mantel-Cox) test p-value for Txnrd1High (n=888) and Txnrd1Low (n=886), (c) Kaplan-Meier survival curve for recurrence-free survival generated by pooling the data for all the datasets after quantile normalization along with Log-Rank (Mantel-Cox) test p-value. The cumulative mean survival for Txnrd1High (n=903) and Txnrd1Low (n=807) cohorts is shown below the survival curve, (d) A forest plot shows pooled hazard ratio, 95% CI, weight assignment and heterogeneity obtained by multivariate analysis for datasets included in this study, (e) Meta-analysis for log-transformed relative risk along with associated statistical parameters for metastasis free survival. Adapted from Patwardhan et al., Heliyon, 2024. 10(6):e27011

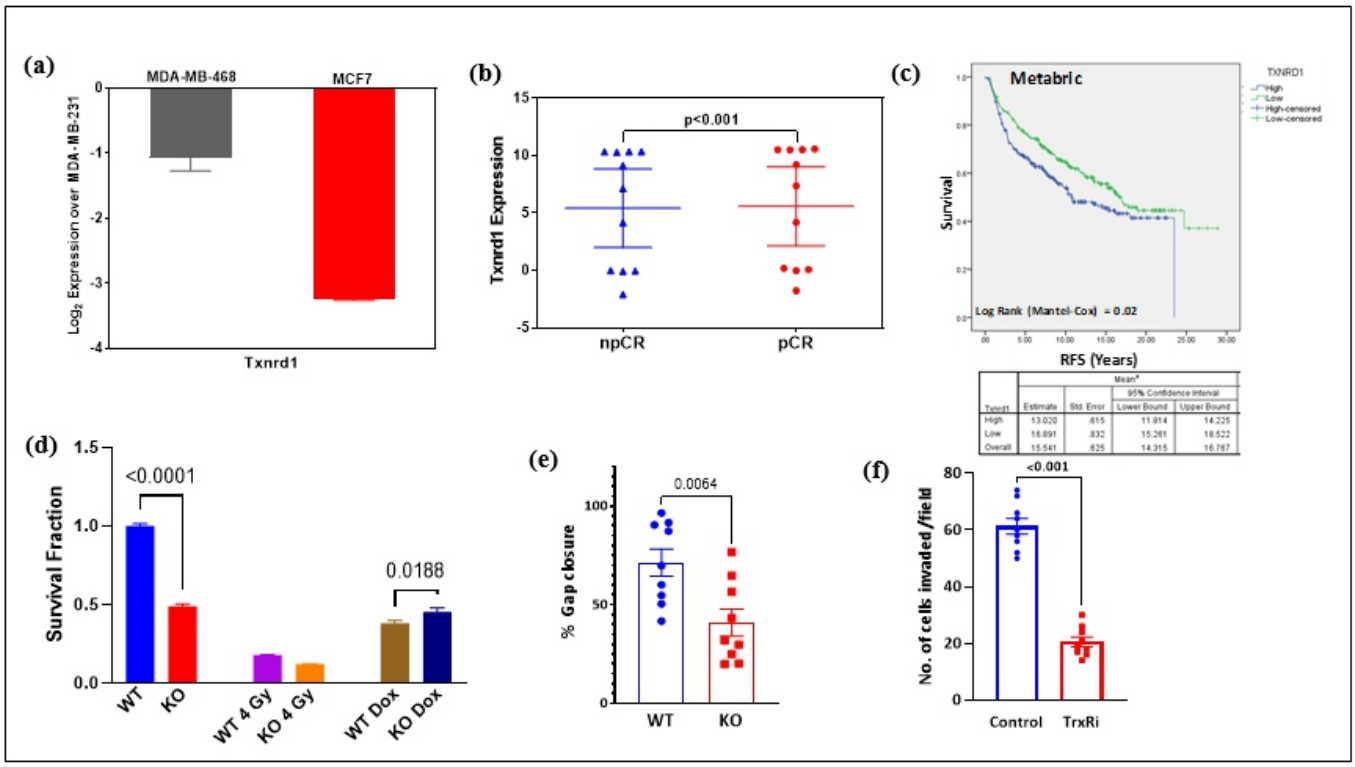
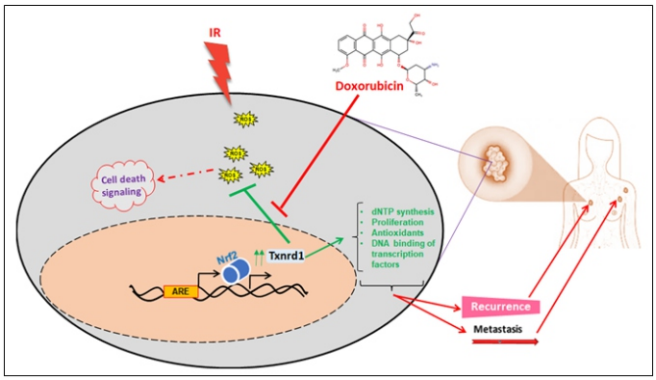


Fig.3: (a) Bar graph shows Log2 expression of Txnrd1 in MCF7 and MDA-MB-468 cells relative to MDA-MB-231, (b) Mean Txnrd1 expression between breast cancer patients with or without pCR, (c) Kaplan-Meier survival curve for recurrence-free survival of breast cancer patients after radiotherapy between Txnrd1 high or low cohorts. Significance is calculated by the Log-Rank(Matel-Cox) test, and the cumulative mean survival of Txnrd1High (n=198) and Txnrd1Low (n=166) cohorts is shown below the survival curve, (d) The bar graph shows survival fraction calculated from clonogenic assay performed with wild type and Txnrd1 depleted MDA-MB-231 cells treated with or without Doxorubicin or radiation 4 Gy, (e) The bar graph shows percent gap closure for wound healing scratch assay at 0 h and 24 h for wild type Txnrd1 depleted cells, (f) The bar graph shows number of cells invaded per field from Transwell Matrigel invasion assay performed with MDA-MB-231 cells treated with or without pharmacologic inhibitor of TrxR. Adapted from Patwardhan et al., Heliyon, 2024. 10(6):e27011

our findings about metastatic behaviour in Txnrd1^{High} cohort. Together these findings reveal that, Txnrd1 over-expressing patients are at elevated risk of local or distant disease recurrence, which enhances the probability of death. However, several other contributing factors will ultimately determine the risk of mortality.

Patients with over-expression of Txnrd1 exhibit better prognosis for chemotherapy but not radiotherapy

Patients achieving pCR after taxane-anthracycline neoadjuvant chemotherapy exhibited significantly elevated Txnrd1 gene expression at $p < 0.05$ compared to the non-pCR group (Fig.3(b)). Higher Txnrd1 expression is consistently associated with pCR across datasets, suggesting its potential as a predictive marker for chemotherapy response. Although radiotherapy exhibits superior tumor control and better QoL with longer event-free survival, not all patients benefit from radiotherapy due to the activation of radioresistance pathways post-radiotherapy. Txnrd1^{High} breast cancer patients undergoing radiotherapy (n=292) exhibited a mean recurrence interval of 13.02±0.61 (95% CI-11.81; 14.22) years as compared to Txnrd1^{Low} cohort (n=244) which showed mean recurrence interval of 16.89±0.23 (95% CI- 15.26; 18.52) years with log-rank (Mantel- Cox) p-value, 0.019 (Fig.3©). Thus, Txnrd1 over-expressing breast cancer patients who have undergone radiotherapy exhibited significantly shorter recurrence intervals as compared to those with low expression of Txnrd1.



Scheme 1: Txnrd1 over-expression is associated with recurrence, metastasis and therapy response in breast cancer Adapted from Patwardhan et al., Heliyon, 2024. 10(6):e27011

Conclusion

In summary, Txnrd1 over-expression is associated with higher grade, hormone receptor negative, HER2 positive, larger tumors of aggressive subtype and exhibit significant hazard for shorter breast cancer-specific and overall survival. Txnrd1 over-expressing breast cancer patients are at elevated risk of early recurrence and metastasis. Further, Txnrd1^{High}-expression cohort of breast cancer patients exhibit a pathologic complete response to neoadjuvant chemotherapy but are poor responders to radiotherapy (Scheme 1). Hence, in Txnrd1 over-expressing breast cancer patients, radiotherapy can be combined with taxane-anthracycline chemotherapy for better therapeutic outcomes [11].

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